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The Dac-tag, an affinity tag based on penicillin-binding protein 5

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ABSTRACT

Penicillin-binding protein 5 (PBP5), a product of the *Escherichia coli* gene *dacA*, possesses some β-lactamase activity. On binding to penicillin or related antibiotics via an ester bond, it deacylates and destroys them functionally by opening the β-lactam ring. This process takes several minutes. We exploited this process and showed that a fragment of PBP5 can be used as a reversible and monomeric affinity tag. At ambient temperature (e.g., 22 °C), a PBP5 fragment binds rapidly and specifically to ampicillin Sepharose. Release can be facilitated either by eluting with 10 mM ampicillin or in a ligand-free manner by incubation in the cold (1–10 °C) in the presence of 5% glycerol. The "Dac-tag", named with reference to the gene *dacA*, allows the isolation of remarkably pure fusion protein from a wide variety of expression systems, including (in particular) eukaryotic expression systems.

For protein purification, ectopically expressed proteins are normally tagged to allow the use of affinity chromatography. Most of the commonly used tags are optimized for bacterial expression. The GST-tag, which exploits the interaction between glutathione *S*-transferase (GST)[1] and glutathione (GSH), is probably the most commonly used affinity tag for protein purification [1,2]. This tag is more powerful in bacteria than in eukaryotic cells because eukaryotic cells contain several isoforms of endogenous GST and also GSH, which interfere with the purification of ectopically expressed GST-tagged proteins. The maltose-binding protein (MBP)-tag provides very good selectivity and is often used to improve solubility of target proteins. The drawback of this tag is its size of just over 40 kDa [3]. In contrast, the short polyhistidine-tag consists of an amino acid repeat of 6 or more histidine residues and is bound to an immobilized metal, such as Ni^{2+}-nitrilotriacetic acid (NTA) Sepharose or TALON resin, and eluted with high concentrations of imidazole [4–7]. Although the polyhistidine-tag works reasonably well for bacterial expression, particularly with highly expressed proteins, its use in eukaryotic cells is restricted to highly expressed proteins due to the relatively low selectivity of the affinity media. A number of proteins contain "natural" His-tags within their amino acid sequence. Metal resins also act as anion exchangers and bind to a variety of contaminants. Furthermore, subsequent removal of the eluent imidazole is imperative for most downstream applications, introducing a second purification step, which may reduce yields. Other popular tags include epitope tags, which are recognized by specific antibodies, such as the FLAG-tag [8], the Myc-tag [9], and the hemagglutinin (HA)-tag [10]. They are short, inert, and suitable for immune detection by Western blotting and for small-scale purification, but not for medium- or large-scale protein purification, because the eluents are peptides or antibodies that are expensive and contaminate the samples. Other proprietor tags, such as the Strep-tag, the S-tag, and the Halo-tag, have been introduced during the past couple of years. For the isolation of very pure native fusion proteins, tandem affinity purification (TAP) methods have been introduced. These methods share a principle by using two or three different tags (e.g., GST, His_6, Ca^{2+}-binding domains, streptavidin, protein G-binding domains, HA, Myc), sometimes separated by a protease cleavage site [11–13]. TAP-tag methods are more complex and more expensive than a one-step procedure. They are also more difficult to scale up, and yield is diminished by the need for at least two chromatographic steps.

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[1] *Abbreviations used:* GST, glutathione *S*-transferase; GSH, glutathione; HA, hemagglutinin; TAP, tandem affinity purification; PBP, penicillin-binding protein; PBP5, penicillin-binding protein 5; NHS, *N*-hydroxysuccinic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; cDNA, complementary DNA; TEV, tobacco etch virus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; LB, Luria–Bertani; SD, synthetic deficient; CHC, clathrin heavy chain; PR, peroxiredoxin-1; fc, final concentration.

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When working with proteins purified from eukaryotic expression systems, we noticed that many of the currently available tags have limitations, especially when we aimed for high protein purity at low expression levels. To overcome this problem, we set out to develop a new tag using a ligand–enzyme pair that is alien to eukaryotic cells in the hope that this approach would yield a tag with great specificity and purity. In particular, we exploited the interaction between a penicillin and an *Escherichia coli* penicillin-binding protein.

A unique feature of bacterial cells is the composition of their cell wall, which, in contrast to plants and fungi, is made up of peptidoglycan chains. Transpeptidases crosslink these chains, creating tetrapeptide bridges made up of L- and D-amino acids. The extent of crosslinking is regulated by carboxypeptidases, which are therapeutic targets of β-lactam antibiotics, encompassing the penicillins and derivatives thereof. The penicillin drug targets are referred to as penicillin-binding proteins (PBPs), and *E. coli* naturally expresses 11 PBPs: PBP1a, PBP1b, PBP1c, PBP2, PBP3, PBP4, PBP4b, PBP5, PBP6, PBP6b, and PBP7 [14]. They also have names that describe their catalytic activity. One of them, PBP5 or D-alanyl-D-alanine carboxypeptidase fraction A, is encoded by the *dacA* gene in *E. coli*. PBP5 (acc. no. P0AEB2) has been very well characterized. Many of its biochemical properties have been elucidated, and the crystal structure has been solved [15–20]. Amino acids 37 to 297 form the catalytic domain. This is followed by a finger-like domain made up of β-sheets and a membrane attachment sequence at the extreme C terminus. The N-terminal 36 amino acids are probably not involved in the catalytic mechanism. PBP5 possesses β-lactamase activity and deacylates penicillin G with a half-life of approximately 9 min [16,17,20,21]. This implies that the binding to penicillin derivatives is reversible, which in turn makes the catalytic domain of PBP5 a candidate for a reversible protein tag. Furthermore, PBP5 exists as a monomer, which has certain advantages for a protein tag, for example, when protein dimers or oligomers need to be purified.

Materials and methods

Consumables

If not stated otherwise, all chemicals were obtained from VWR International and purchased at the highest available quality. GSH Sepharose and *N*-hydroxysuccinic acid (NHS)-activated Sepharose were purchased from GE Healthcare Life Sciences (UK). Ampicillin was obtained from Calbiochem (UK). Prestained protein marker SeeBlue Plus, Cellfectin II, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), gentamycin, and oligonucleotides were obtained from Invitrogen. Insect medium, Insect Express, was obtained from Lonza. The marker used in Fig. 4E was Marker II from Peqlab (Germany). For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the SciePlas 20 × 20-cm system was used. Restriction enzymes were obtained from Fermentas, agarose was from Melford, and protein stain was Instant Blue from Expedeon.

Cloning of mammalian expression constructs

For PBP5-GFP, the *dacA* fragment Met37-Asp392 of acc. no. AP_001281 was cloned from genomic DNA of *E. coli* strain JM109 by polymerase chain reaction (PCR) using the primers ATCCGCTAGCCACCATGATCCCGGGTGTACCGC and GTAAGCTTGGGCCCCTGGAACAGAACTTCCAGATCAATGATTTTGCCGAAGAAGTACC so that a site for PreScission Protease and a multicloning site were added. For the shorter Dac-GFP, the *dacA* fragment Met37-Pro297 was cloned by PCR using ATCCGCTAGCCACCATGATCCCGGGTGTACCGC and GATAAGCTTTGGGTTAACGGTTTCAAAGAAACG. The PCR fragments were cloned into the *Nhe*I and *Hin*dIII sites of the pEGFP-N1 vector. To express Dac-SPAK, the region coding for amino acids 37 to 297 was amplified by PCR and cloned into the pcDNA3.1 vector as a *Hin*dIII-*Not*I fragment upstream of a multicloning site to clone other complementary DNAs (cDNAs) downstream in frame with it. The cloning of SPAK-STE20/SPS1-related proline/alanine-rich kinase (NCBI acc. no. AF099989) has been described previously [22]. The full-length SPAK cDNA has been cloned C-terminally from the Dac-tag using *Bam*HI-*Eco*RV-*Not*I three-way ligation. To express GST-SPAK, the cDNA was cloned into the pcDNA3.1 vector with the same cloning strategy.

Cloning of PBP5 amino acids 37 to 297 into pET24a

pET24a Dac was created by amplifying the Dac insert from the mammalian Dac-GFP vector and subcloning into the *Nde*I and *Not*I sites of pET24a.

Cloning of pFB-Dac-GFP construct for baculovirus

PBP5 residues 37 to 297 with a modified start (MSVPG) were amplified using CGGTCCGAAACCATGTCCGTGCCGGGTGTACCGCAGATCGAT and GCGGATCCTGGGTTAACGGTTTCAAAGAAACGGAAGC to provide an *Rsr*II-*Bam*HI insert. The GFP was amplified using primers GGATCCGTGAGCAAGGGCGAGGAGCTGTTC and GAATTCTTACTTGTACAGCTCGTCCATGCCGA with a 5′ *Bam*HI and 3′ *Eco*RI site (plus stop codon), and then the two Dac and GFP fragments were ligated to the pFB backbone in a three-way *Rsr*II-*Bam*HI-*Eco*RI ligation.

Cloning of pFB-Dac-ATG7 construct for baculovirus

For a pFast-Bac-Dual-Dac-TEV-parent vector, a *Bgl*II-*Eco*RI flanked Dac-tobacco etch virus (TEV) fragment was amplified from a modified Dac clone (N terminus changed from MIPGVP to MSAIGVP using GTACATGTCTGCAATCCCGGGTGTACCGCAC) using GTAGATCTCAACATGTCTGCAATCCCGGGTTACCG and ACGAATTCCGATCGGGATCCGCCCTGAAAATACAGGTTTTCTGGGTTAACG and then subcloned into the *Bam*HI-*Eco*RI sites of pFBDual to give the final vector. cDNA coding for human ATG7 isoform b (NP_001129503.2) was amplified from IMAGE consortium EST 3504204, cloned into vector pSC-B (Stratagene), and fully sequenced. This sequence was then converted to the isoform a sequence (NCBI acc. no. NM_006395.2) by PCR mutagenesis. Following conversion to the isoform a sequence, the insert was subcloned into pFastBac-Dual-Dac-TEV using restriction sites *Bam*HI/*Not*I to generate a construct for expression of Dac-TEV-ATG7 isoform a.

Cloning of pFB dual His_6-TEV-Hrt1/Dac-TEV-Cdc53 plasmid for baculovirus

The coding region for yeast (*Saccharomyces cerevisiae*) *hrt1* (NCBI acc. no. NM_001183387.1) was amplified from yeast genomic DNA, adding an *Xho*I site with His_6 and a TEV cleavage site in the 5′ primer and an *Nhe*I site in the 3′ primer. The full-length PCR product was cloned into pSC-B (Stratagene) and sequenced. The His_6-TEV-Hrt1 was subcloned into pFastBac-Dual (Invitrogen) as an *Xho*I/*Nhe*I insert. The coding region for yeast *cdc53* (NCBI acc. no. NM_001180191.1) was also amplified from yeast genomic DNA, adding *Bam*HI and *Not*I restriction sites in the 5′ and 3′ primers, respectively. The full-length PCR product was then cloned into pSC-b and sequenced. *Cdc53* was then subcloned from this vector into pEBG-Dac, a modified pEBG 2T vector in which the GST-tag has been replaced with a Dac-tag as a *Bam*HI/*Not*I insert. The Dac-Cdc53 expression cassette was amplified from this vector,

cloned into pSB-B, and sequenced. Dac-Cdc53 was then subcloned into the pFastBac Dual His_6-TEV-Hrt1 vector detailed above as an *Eco*RI/*Not*I insert for baculovirus expression. All PCR reactions were carried out using KOD HotStart DNA Polymerase (Novagen).

Cloning and transformation of dacA Dictyostelium expression construct

DacA (37–297) was excised from Dac-GFP with *Sma*I and *Hin*dIII and cloned into *Bam*HI (blunt) and *Hin*dIII digested pDV-NTAP-CYFP [23] to create pDV-NABP-CYFP where an N-terminally Dac-YFP is expressed under control of the actin 15 promoter of *Dictyostelium discoideum. D. discoideum* wild-type AX2 cells were transformed with 10 μg of pDV-NABP-CYFP [24] and selected for 7 days with 20 μg/ml G418.

HEK293 cell culture, transfections, and extracts

HEK293 cells were grown in 10-cm dishes in DMEM, supplemented with 10% fetal calf serum (FCS) and 0.5 mg/ml gentamycin, at 37 °C in an atmosphere containing 5% CO_2. Then, cells were transfected using the calcium phosphate method. Briefly, for each dish of cells, 5 to 10 μg of DNA was mixed with 61 μl of 2 M $CaCl_2$ and made up to 500 μl with H_2O. Then, 500 μl of 2 × HBS (50 mM Hepes [pH 7.4], 280 mM NaCl, and 1.5 mM $Na_2HPO_4 \times 2H_2O$) was aliquoted into a 15 ml vial. The DNA mix was added dropwise, with the mix being constantly vortexed. This mix was then carefully dropped onto the dishes, and they were left to become transfected overnight. The cells were left for 3 days before being collected in PBS, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM ethyleneglycoltetraacetic acid (EGTA) and then sedimented by centrifugation. The sediments were resuspended in 8 volumes of 50 mM Tris–HCl (pH 7.5), 0.2% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM Pefabloc, and 20 μg/ml leupeptin and then incubated for 5 min on ice and clarified by centrifugation for 6 min at 15,000*g* at 4 °C. Other buffer systems such as HOPES–NaOH, phosphate buffer, and MOPS–NaOH were also used.

Preparation of baculovirus and infection of Sf21 cells

FastBac constructs were transformed into DH10Bac cells and grown under antibiotic selection on plates containing X-gal. White clones, which indicated that bacmids had formed, were picked and after a second round of selection were used to prepare bacmid DNA. Bacmid DNA was transfected into Sf21 cells using Cellfection II (Invitrogen), and the transfection was left for a week to prepare a P0 virus. The cells and the medium were harvested and separated by centrifugation. The medium was used to infect fresh Sf21 cells at a dilution of 1:50. After 4 or 5 days, the cells were collected and used for protein purification. The medium was kept and used as a P1 virus stock.

Preparation of D. discoideum extracts

Cells, grown from a pool of transformants, were harvested by centrifugation at 800*g* for 2 min and washed twice with 16 mM KH_2PO_4 and 40 mM K_2HPO_4 (pH 6.8), resuspended in 10 mM Tris (pH 7.5), 150 mM NaCl, EDTA-free protease inhibitor cocktail (Roche), and Protease Inhibitor Mix VIII (Merck), and filter-lysed through a 3-μm filter (Whatman). The lysate was clarified by centrifugation for 5 min at 16,100*g*. The protein concentration was adjusted to 2 mg/ml for purification over ampicillin Sepharose.

Preparation of E. coli cell extracts

BL21(DE3) pLysS cells (Promega) were transformed with pET24a Dac-GFP. Overnight culture (5 ml), grown in Luria–Bertani (LB)/kanamycin, was used to inoculate 500 ml of LB/kanamycin. At OD_{600} = 0.7, protein expression was induced by supplementing the medium with 0.5 mM isopropyl-β-D-thiogalactopyranoside and the temperature was adjusted to 30 °C. Cells were collected by sedimentation 18 h after induction, resuspended in 10 volumes of 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM Pefabloc, and 10 μg/ml leupeptin, and sonicated using a Sonics Ultracell sonicator at 30% amplitude. The lysate was cleared by centrifugation at 25,000*g* for 20 min at 4 °C. Extracts were diluted to 3 mg/ml for affinity chromatography.

Preparation of ampicillin Sepharose

Ampicillin Sepharose was prepared by coupling ampicillin (0.2 M fc) to NHS-activated Sepharose. Briefly, NHS-activated Sepharose was washed in 1 mM HCl. The Sepharose was equilibrated in 0.1 M $NaHCO_3$, mixed with an equal volume of 0.4 M ampicillin in 0.1 M $NaHCO_3$ (pH 7.5), and incubated for 4 h at 22 °C. The unbound ampicillin was washed away with 20 volumes of 0.1 M Tris–HCl (pH 7.5). The Sepharose was washed with 20 volumes of 20% EtOH and stored at 4 °C in 20% EtOH. Ampicillin Sepharose stored at 4 °C in 20% EtOH is stable for at least 6 months.

Purification of PBP5 fusion proteins over ampicillin Sepharose

Prior to use, ampicillin Sepharose was washed three times with 10 volumes of H_2O. Cell extracts were diluted to 2 to 4 mg/ml. The cell extract was brought to ambient temperature (20–23 °C) and added to the ampicillin Sepharose. The extract was mixed for 50 min with ampicillin Sepharose at ambient temperature in a tube roller. The Sepharose was sedimented by centrifugation and washed three to five times with wash buffer (40 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 0.03% Brij-35) at ambient temperature. In Fig. 3B, various concentrations of NaCl were used. Small sediments (<50 μl) in 1.5-ml reaction tubes were washed four times with 1 ml of buffer. Larger sediments were washed in 15-ml centrifuge tubes. The total washing time did not exceed 15 min in order to minimize losses. Proteins were released by allowing the β-lactamase activity of PBP5 to cleave the bond with ampicillin under conditions where PBP5 cannot easily bind to another immobilized ampicillin. This was achieved by either competing with mobile ampicillin or cooling the sample on ice. Any release or elution buffer contained 100 mM NaCl, a mild detergent, and 5% glycerol (e.g., 40 mM Tris–HCl [pH 7.5], 0.1 M NaCl, 0.03% Brij-35, and 5% glycerol). After elution or release, the proteins were analyzed by SDS–PAGE. Proteins were stained for 1 h with Instant Blue (Expedeon), followed by washing in water. For Fig. 4B, we performed a silver stain using the Silver Staining Kit from GE Healthcare Life Sciences.

Preparation of yeast cell extract

To generate a yeast expression plasmid, 3 × HA-9 × His-Dac-tag was cloned using *Bam*HI and *Eco*RI sites into the yeast expression vector pRD54 under the Gal1–10 promoter. For expression of tagged Rub1p, the Rub1p cDNA was inserted into this vector using *Eco*RI/*Xho*I restriction sites and transformed into yeast deleted for endogenous Rub1p. Rub1p deleted yeast cells were transformed with a galactose-inducible expression vector containing Dac-Rub1p. A 100-ml *S. cerevisiae* starter culture was grown in synthetic deficient (SD)-raffinose–Ura medium at 30 °C overnight. In the morning, the culture was diluted to OD_{600} = 0.2 in 3 L of either

2% SD-raffinose (control cells) medium or 2% SD-galactose (Dac-Rub1p induction) medium and allowed to grow until OD_{600} reached 0.8. The cells were harvested by centrifugation and resuspended in approximately 1 volume of extract buffer (40 mM Tris–HCl [pH 8.2], 0.2% Triton X-100, 1 mM EDTA, and 1 mM EGTA) containing Roche Complete Protease Inhibitor. The slurry was subsequently flash-frozen by dripping it into liquid nitrogen. The kernels were ground in a pre-chilled mortar and pestle in the presence of liquid nitrogen, and the resulting powder was thawed on ice. The extract was subsequently cleared by centrifugation, and total protein concentration was determined by Bradford assay and adjusted to approximately 5 mg/ml with extract buffer for pull-downs using ampicillin Sepharose.

Preparation of images

The stained acrylamide gels were scanned using an Epson Perfection V500 scanner at 150 dpi in color mode. The images were assembled in Adobe Illustrator CS3. These files were opened with Adobe Photoshop and saved in JPEG format. In Fig. 4B, the contrast was markedly enhanced without changing the shape of the contrast curve in order to show proteins, which otherwise would difficult to see. Fig. 5C was created using a Licor Odyssey Imager, saved in grayscale TIFF, and then modified and assembled like the other images in Illustrator.

Results and discussion

Development of Dac-tag as a covalent but reversible tag

For the development of a reversible tag, two aspects of the system needed to be developed. We needed to generate and optimize the protein part of the tag, on the one hand, and the affinity resin, on the other. The latter aspect was straightforward. Ampicillin Sepharose had been made and used to isolate PBPs since at least 1975 [25]. A very useful ampicillin Sepharose can be generated by coupling ampicillin to NHS-activated Sepharose.

For the protein aspect, we identified a suitable fragment of PBP5 and established binding, washing, and elution conditions. Furthermore, we examined purity and yield from several expression systems. To test whether soluble fragments of PBP5 can be expressed in and purified from eukaryotic cells, we cloned fusion proteins with GFP into mammalian and insect expression vectors. A soluble PBP5 fragment encompassing amino acids 37 to 392 followed by a Prescission Protease site was cloned into pEGFP and transiently transfected into HEK293 cells (Fig. 1B). The protein was well expressed, as judged by fluorescence. Cell lysates were incubated for 50 min with ampicillin Sepharose at ambient temperature. The Sepharose was then washed and transferred into spin filters to prevent contamination of the eluate with Sepharose. The Sepharose was incubated three times for 10 min without or with various concentrations of ampicillin, and the eluates were collected. We analyzed recovery of PBP5-GFP by SDS–PAGE followed by Coomassie blue staining (Fig. 1C). Although some protein was recovered in the absence of ampicillin, the addition of 10 mM ampicillin caused the release of practically all of the PBP5-GFP fusion protein. The purity of the fusion protein expressed in HEK293 cells was good, but we noticed two contaminants, which were identified by mass spectrometry as clathrin heavy chain (CHC) and peroxiredoxin-1 (PR). With these transient transfections, we recovered 2 to 5 μg of fusion protein from 1 mg of cell lysate protein or 3 to 8 μg per 10-cm dish.

At low temperatures, wild-type PBP5 does not bind to ampicillin Sepharose efficiently. However, when PBP5-loaded ampicillin Sepharose is exposed to the cold, PBP5 is still active as a β-lacta-

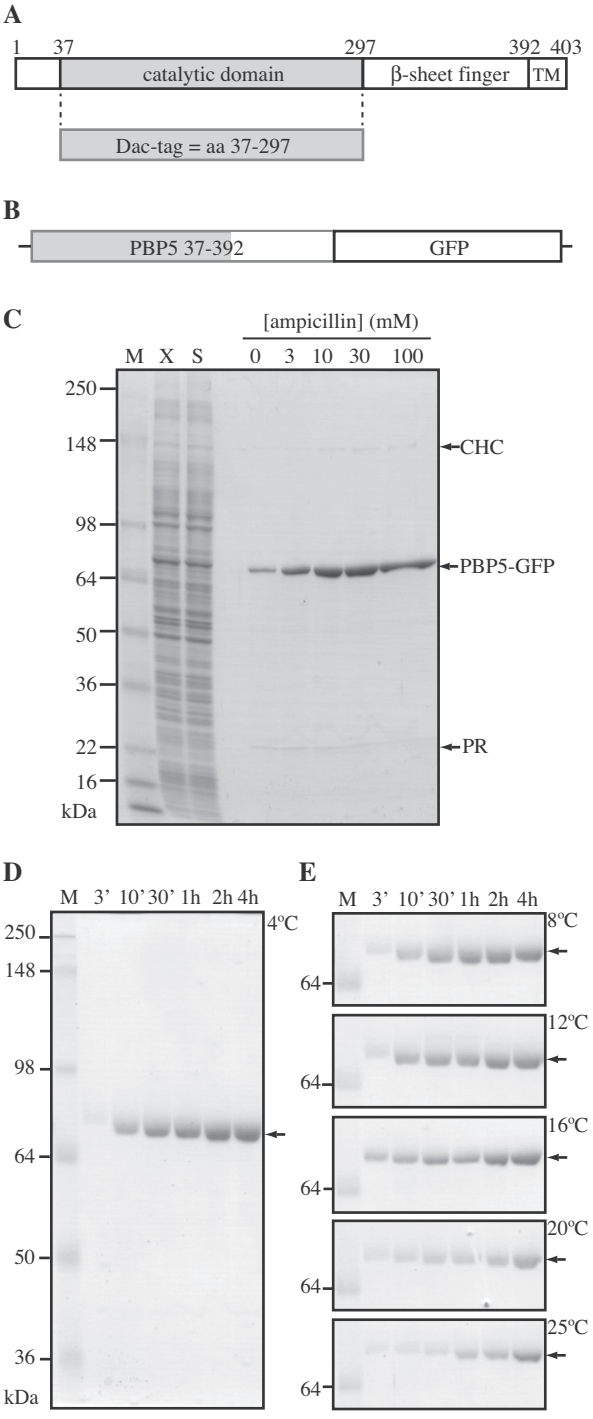

Fig.1. Purification of a PBP5-GFP fusion protein over ampicillin Sepharose. (A) Domain structure of *E. coli* PBP5 with the catalytic domain referred to as "Dac-tag." (B) PBP5 amino acids 37 to 392 cloned into pEGF-N1 yields PBP5-GFP. (C) HEK293 cells were transiently transfected to express PBP5-GFP. This fusion protein was bound to ampicillin Sepharose, washed, and split into five aliquots, which were eluted by incubation with 40 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 0.03% Brij-35, and 0.1% 2-mercaptoethanol without (0) or supplemented with 3, 10, 30, or 100 mM ampicillin. Here, 12 μg of the lysate (X), the supernatant (SN), and all of the eluate were separated by SDS–PAGE (6–20%) and visualized by Coomassie blue staining. PBP5-GFP is indicated with an arrow. (D and E) HEK293 cells were transiently transfected as in panel C. Following expression, PBP5-GFP was captured on ampicillin Sepharose and washed. The Sepharose was split into 36 equal aliquots, which were incubated as indicated for various periods of time at various temperatures with 8 volumes of 40 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 0.03% Brij-35, and 0.1% 2-mercaptoethanol (without ampicillin). The released protein was filtered through a 0.45-μM filter and separated by SDS–PAGE (10%). Panel B shows the whole gel, whereas panel E shows only the region were the PBP5-GFP fusion protein migrates as indicated with an arrow.

mase. This can be used for ligand-free recovery of the fusion protein. We exposed PBP5-GFP-loaded ampicillin Sepharose to a range of temperatures and recovered any released protein at various time points. The protein was then analyzed on SDS–PAGE (Fig. 1D and E). PBP5 is released within 30 min at 4, 8, and 12 °C. At 16 °C or higher temperatures, a delay of protein release can be observed, and this is more noticeable above 20 °C (Fig. 1E). This is not caused by a delayed off-rate given that we can rapidly elute the protein in the presence of ampicillin (Fig. 1C). Rather, above 20 °C PBP5 binds more efficiently back to some of the excess ampicillin on the Sepharose. NHS-activated Sepharose can provide a ligand concentration of up to 15 mM. This is much larger than the protein capacity of the Sepharose, which is 0.135 mM (see below) because it is restricted by molecular crowding. Hence, in the cold, where binding to ampicillin is less efficient, more protein is in the mobile phase and can be recovered. This ligand-free recovery is best achieved by repeated collection of the released protein at 5- to 10-min intervals when an equilibrium has been established. The protein concentration of the cold-released protein is not as high as with ampicillin elution because ampicillin is more effective in preventing the binding of PBP5 to ampicillin Sepharose.

To characterize and optimize the affinity tag further, we cloned a shorter fragment of PBP5 encompassing amino acids 37 to 297. This construct does not express the C-terminal domain of PBP5, which is redundant for the purpose of a tag (Fig. 2A). Furthermore, we changed the sequence at the N terminus of this fragment from MIPG to MSVPG or MSAIPG in order to provide a better start for translation. This shorter fragment of PBP5, notwithstanding any small changes to the N terminus, is now referred to as the Dac-tag. A Dac-GFP fusion was expressed in Sf21 cells. Expression of Dac-GFP was excellent, and the cells of the P1 and P2 passages turned very visibly green. We used this protein to characterize the elution and release properties in more detail. To this end, Dac-GFP-loaded ampicillin Sepharose was distributed into 17 aliquots, which were incubated at 22 °C for 30 min with an elution buffer containing various concentrations of ampicillin. Half maximal elution was achieved at 0.5 mM ampicillin, and optimal elution occurred upward of 10 mM ampicillin (Fig. 2B). We also supplemented the elution and release buffers with 5% (v/v) glycerol, to optimize recovery, because we had noticed earlier that glycerol in the lysis buffer was somewhat detrimental to binding. This explains why some protein eluted in the absence of ampicillin and with very low ampicillin concentrations (Fig. 2B), which does not occur in the absence of glycerol (not shown). We recovered approximately 7.5 mg of protein per milliliter of ampicillin Sepharose, which for a 55-kDa protein gives a resin protein capacity of 135 μM. The purity of the protein was excellent (Fig. 2C). We studied in more detail the effect of the absence or presence of ampicillin on the elution kinetics at 4 °C and at ambient temperature (22 °C) by eluting and collecting aliquots of Dac-GFP-loaded ampicillin Sepharose every 5 min under these conditions (Fig. 2D). The fastest elution occurs with 10 mM ampicillin at 22 °C (Fig. 2D, closed triangles). Elution with ampicillin at 4 °C takes roughly 50% longer (open triangles), indicating slowed down β-lactamase activity. Release at 4 °C in the absence of ampicillin is slower still and might not be complete within a reasonable amount of time (closed circles). Even at 22 °C in the absence of ampicillin, some protein can be recovered over a longer period of time (open circles). This is because in the presence of glycerol after 5 min, there is always some protein in the mobile phase and this is simply washed off. In fact, any reversible tag can be washed off with multiple washes because there is always some protein in the mobile phase.

PBP5 destroys every ampicillin molecule it binds to by opening the β-lactam ring. After a number of cycles, the concentration of effective ampicillin on the resin drops, leading to a reduction in dynamic protein capacity. Therefore, it was necessary to compare the yield from Dac-GFP purifications that had been incubated with ampicillin Sepharose for various periods of time in order to establish a useful binding period. As shown in Fig. 2E, yield is optimal when protein is contacted with the ampicillin Sepharose for between 40 min and 2 h. As expected, the yield does drop with longer incubations. Our standard contact time is 50 min, which is suitable for proteins expressed at high or low levels. In line with this observation, and due to the destruction of the immobilized ligand, we observed a notable drop (up to 50%) of binding capacity when the ampicillin Sepharose was recycled after a purification with saturating amounts of Dac-GFP (data not shown). Hence, recycling of the ampicillin Sepharose is not useful.

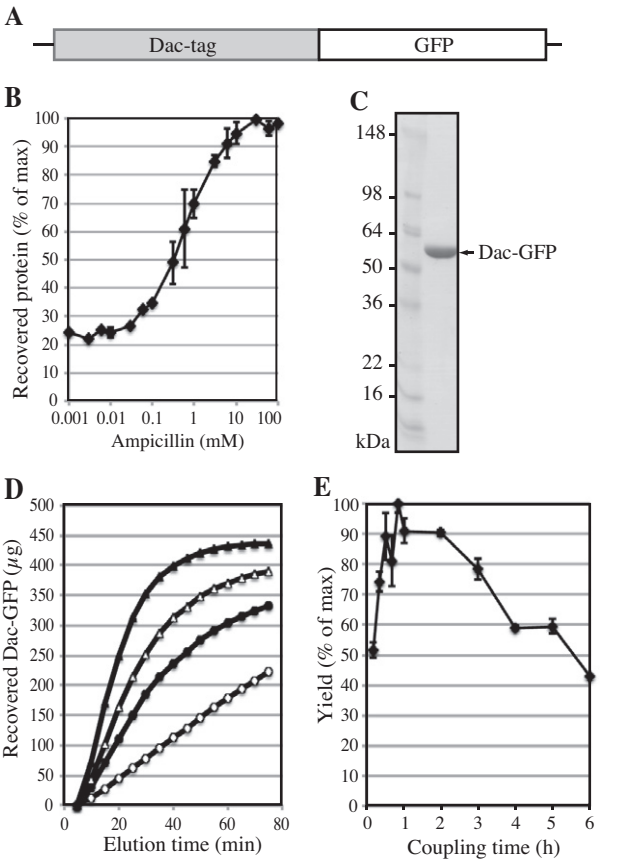

Fig.2. Binding and elution properties of PBP5 (aa 37–297). (A) PBP5 amino acids 37 to 297 (Dac-tag) with a modified N terminus (MSVPG . . .) was cloned N-terminally of GFP into pFastBac Dual for the production of a baculovirus. (B) Using this virus, Dac-GFP was expressed in Sf21 cells and captured on ampicillin Sepharose. For elution, 25 μl aliquots were incubated for 30 min at 22 °C with 50 μl of 40 mM Tris (pH 7.5), 5% glycerol, 150 mM NaCl, and 0.03% Brij-35 supplemented with various ampicillin concentrations (1 μM to 100 mM). Aliquots were taken to measure the protein concentration and yield. (C) Here, 3 μg of the recovered Dac-GFP was separated on a gradient gel to estimate protein purity. (D) For each curve, Dac-GFP cell extract was captured on 150 μl of ampicillin Sepharose, washed, split into three aliquots, and transferred to 0.45-μM spin filters. For elution, 50 μl of release buffer was added. Every 5 min, the filters were spun, the collected protein was taken, and the Sepharose was immersed in another 50 μl of release buffer. The release buffers were 40 mM Tris (pH 7.5), 5% glycerol, 100 mM NaCl, and 0.03% Brij-35 at 22 °C (open circles), at 4 °C (closed circles), with 10 mM ampicillin at 22 °C (closed triangles), and with 10 mM ampicillin at 4 °C (open triangles). The protein recovered in each step was measured. The curves show accumulated recovered protein. (E) For each data point, 2 × 25 μl of ampicillin Sepharose was incubated with 1.5 mg of Dac-GFP cell extract for various periods of time (10 min to 6 h). The Sepharose was then collected and washed, all protein was eluted and measured, and the results were plotted as a function of time. The yield was normalized against the 50-min time point yield, which was maximal.

We next examined whether protein purity and yield were much affected by the NaCl concentration in the wash buffer. Dac-GFP was expressed in HEK293 cells (Fig. 3A) and bound to ampicillin Sepharose. The Sepharose was washed with 50 mM Tris–HCl (pH 7.5), 0.03% Brij-35, and five different concentrations of NaCl (Fig. 3B). Although in the absence of NaCl in the wash buffer the fusion protein was relatively pure, excellent purity was obtained when the Sepharose was washed with 125 mM NaCl. With higher NaCl concentrations, the yield was somewhat diminished. This experiment shows the high selectivity of ampicillin Sepharose for PBP5.

To determine whether the composition of the buffer system had an influence on capture efficiency, we compared different buffers for the Dac-GFP pull-downs using 50 mM Tris–HCl (pH 7.5), Mops (pH 7.5), Hepes (pH 7.5), or phosphate buffer (pH 7.5) as buffer in the respective lysis, wash, and release buffer. Prior to this, all experiments had been carried out in Tris–HCl buffer systems. There were no noteworthy differences among four side-by-side purifications, demonstrating that the tag performs equally with a wide range of different buffer systems (Fig. 3C). Taken together, our results demonstrate that a fragment of PBP5 can be used as a tag for the expression and purification of proteins from mammalian and insect expression systems.

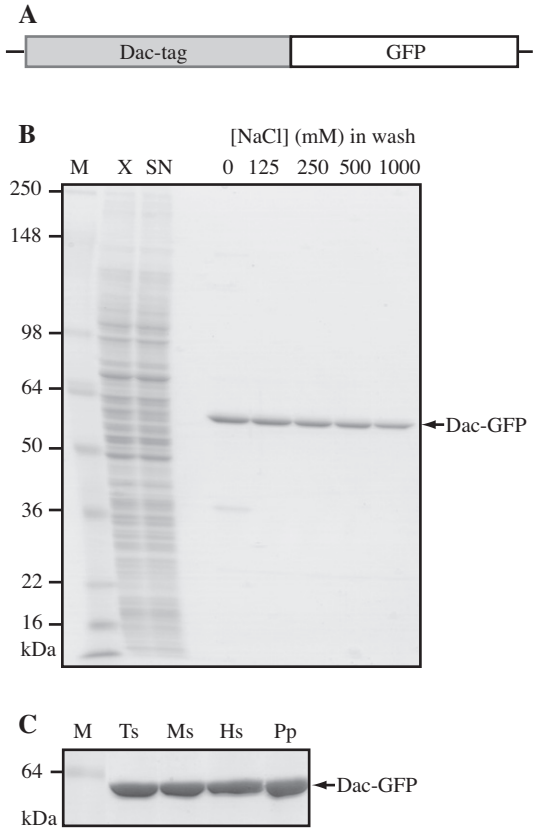

Fig.3. Effect of [NaCl] in the wash buffer and different buffer systems on the performance. (A) PBP5 amino acids 37 to 297 (Dac-tag) was cloned into pEGFP-N1 to express Dac-GFP in human cells. (B) Dac-GFP, expressed in HEK293 cells, was captured on ampicillin Sepharose, which was then washed with wash buffers containing various NaCl concentrations. Dac-GFP was then released and analyzed by SDS–PAGE and protein staining. (C) Dac-GFP from transfected HEK293 cells was captured, washed, and released as described in panel B. The four preparations differed only in the buffer systems used during the procedure. Ts indicates that all solutions were buffered with 40 mM Tris–HCl, Ms (Mops), Hs (Hepes), and Pp (phosphate buffer), all at pH 7.5.

Use of Dac-tag in many cell types

We next examined whether the Dac-tag purification system can be applied to other cell systems. We created expression plasmids for use in *S. cerevisiae*, *D. discoideum*, and *E. coli* (BL21). We also extended the range of plasmids for *Spodoptera frugiperda* because we believed that the insect expression system is particularly needy of a monomeric tag with high selectivity.

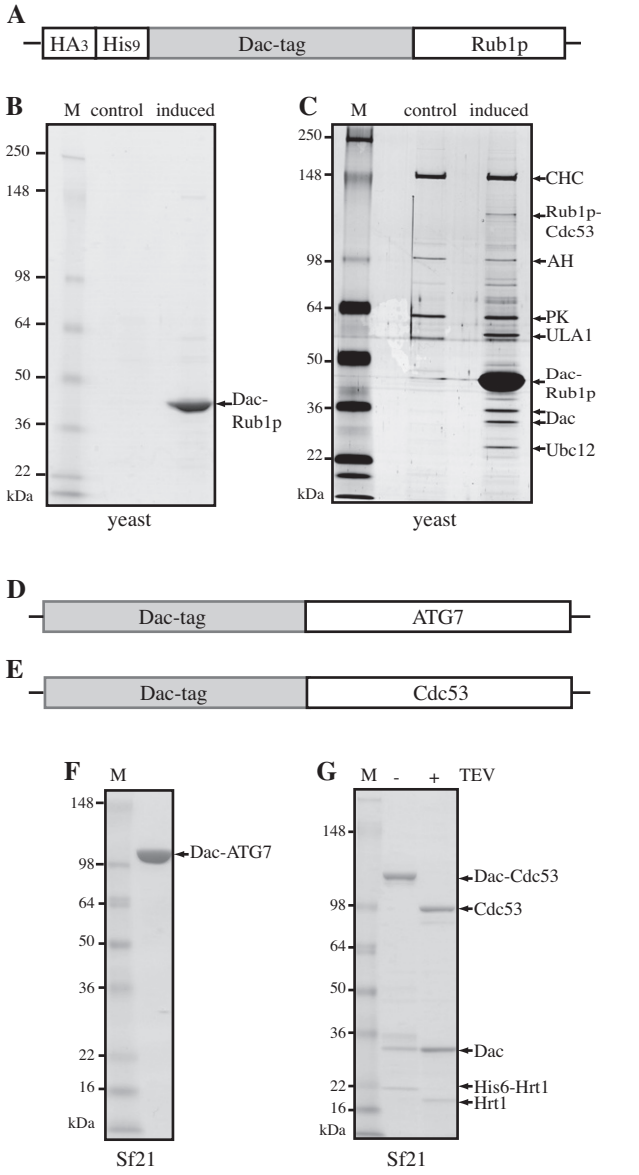

Fig.4. Dac-tag purifications from yeast and insect expression systems. (A) A galactose-inducible yeast vector based on pRD54 was created to express 3 × HA-His_9-Dac-Rub1p. (B) *S. cerevisiae* was transformed with this vector. Expression was induced with galactose (induced) or left noninduced (control). After incubation overnight, the cells were collected, protein extracts were prepared, and Rub1p was purified over ampicillin Sepharose, separated by SDS–PAGE, and stained with Coomassie blue. (C) Same as in panel B except that proteins were visualized with silver stain. Some of the proteins that were identified by mass spectrometry are indicated. (D and E) Vectors based on pFastBac Dual were produced to generate bacmids and baculoviruses for the expression of Dac-TEV-ATG7 (D) or Dac-TEV-Cdc53/His_6-TEV-Hrt1 (E) in Sf21 cells. (F) Dac-TEV-ATG7 was expressed in Sf21 cells. The protein of the P2 cells was purified over ampicillin Sepharose. Here, 3 μg was separated on SDS–PAGE and stained. (G) Dac-TEV-Cdc53/His_6-TEV-Hrt1 was expressed in Sf21 cells. The protein of the P2 cells was purified over ampicillin Sepharose. Some was digested with TEV protease to remove the Dac-tag and the His_6-tag (+). The protein was separated by SDS–PAGE and stained.

For *S. cerevisiae*, we decided to express an N-terminally Dac-tagged version of the ubiquitin-like protein Rub1p (Fig. 4A–C). Rub1p becomes conjugated to yeast cullins via a heterodimeric E1 activating enzyme consisting of the two proteins Ula1p and Uba3p and the conjugating enzyme Ubc12 (neddylation). The major substrate for neddylation is the yeast cullin 1 homologue Cdc53. We examined whether the Dac-tag purification system could be useful for identifying proteins in yeast that become covalently modified by Rub1p. To this end, N-terminally Dac-tagged Rub1p (Dac-Rub1p) was cloned into a galactose-inducible expression vector (Fig. 4A) and transformed into yeast lacking endogenous Rub1p. Dac-Rub1p was induced by growing the cells in medium containing 2% galactose as a carbon source, whereas the control cells were kept noninduced by growth in 2% raffinose medium. After induction, cell extracts were prepared and Dac-Rub1p was captured on ampicillin Sepharose, washed extensively, and released. We analyzed the purified proteins by SDS–PAGE, followed by protein staining with Coomassie blue (Fig. 4B) or silver (Fig. 4C). We treated the extracts from the noninduced cells identically to the induced cells. As shown in Fig. 4B and C, Dac-Rub1p was efficiently purified from the induced cells. In addition to free Dac-Rub1p, there were multiple bands that were specifically purified from the induced extract. Some of these may represent Rub1p modified substrates. Indeed, one of the bands was tentatively identified by mass spectrometry as a mix of Rub1p and Cdc53, the major known Rub1p substrate in yeast (Fig. 4C). Other identified proteins included the E2 conjugating enzyme for Rub1p, Ubc12, and one of the two E1 activating enzyme subunits, ULA1 (Fig. 4C), clearly demonstrating that the Dac-tag can be used to identify specific protein binding partners in budding yeast. With regard to contaminants in the yeast system, we identified yeast CHC, aconitate hydratase, pyruvate kinase, and a number of other proteins at very low levels in both the Dac-Rub1 induced and noninduced cultures (Fig. 4C).

To further test the Dac-tag in Sf21 cells, we created baculoviruses for ATG7 (Fig. 4D), the E1 for the autophagy system and for the coexpression of Dac-TEV-Cdc53 with His_6-TEV-Hrt1 (Fig. 4E), and we infected Sf21 cells with a ratio of 1 virus per cell. After 3 days, we collected the cells and purified the proteins. Dac-ATG7 expressed extremely well and reached levels of 60 mg/L. The protein was purified to homogeneity (Fig. 4F). The expression level of Dac-TEV-Cdc53 was much lower (1.2 mg/L), but the quality of the protein was good (Fig. 4G). We removed the Dac-tag by cleavage with TEV protease and obtained excellent separation from the tag (Fig. 4G), which was subsequently removed by size exclusion chromatography (not shown). This strategy also works very well for the purification of human cullin/Rbx complexes (not shown).

The social amoeba *D. discoideum* is notorious for expressing large amounts of very active proteases, and protein purification from these cells is a particular challenge. To determine whether the Dac-tag can be used in this system, we created a Dac-YFP expression plasmid (Fig. 5A) and transformed *Dictyostelium* cells, which expressed the fusion protein and displayed yellow fluorescence. Using a high concentration of protease inhibitors in the lysis buffer and wash buffer, we isolated hundreds of micrograms of the pure Dac-YFP fusion protein over ampicillin Sepharose. We analyzed a 4-μg aliquot on SDS–PAGE (Fig. 5C).

We next examined whether the Dac-tag might be useful for expression in *E. coli* BL21 cells. To this end, we created plasmids based on the pET vector range. We selected plasmids that confer kanamycin resistance because the β-lactamase of an ampicillin-resistant vector would quickly destroy the affinity ligand. Expression of the Dac-tag starting with MIPG was very poor. MIPG provides a bad translation start. Therefore, we simply subcloned PBP5 amino acids 37 to 297 into pET24a, so that it starts with a His_6-tag (Fig. 5B). This protein was expressed at high levels,

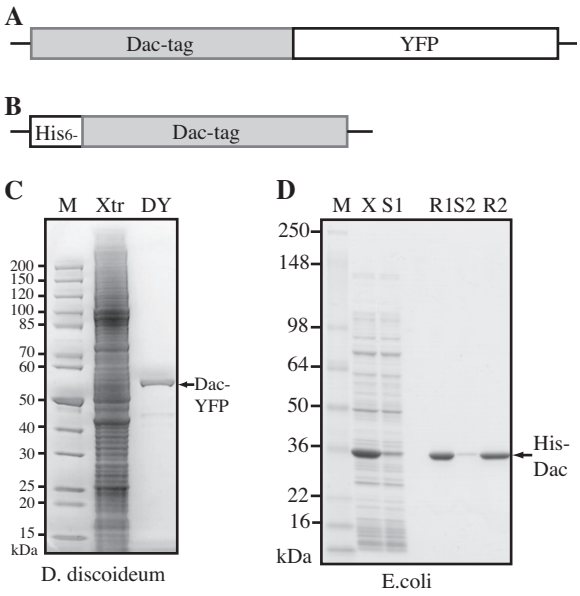

Fig.5. Dac-tag expression in and purification from *D. discoideum* and *E. coli*. (A) The Dac-tag was cloned into the *D. discoideum* expression vector pDV-NTAP-CYFP to express Dac-YFP. (B) The Dac-tag was cloned into pET24a to express His_6-Dac. (C) *D. discoideum* cells were transformed with the expression vector encoding Dac-YFP as in panel A. The cells were grown under selective pressure and harvested, and protein extracts were prepared. Dac-YFP (DY) was isolated by ampicillin affinity chromatography and analyzed by SDS–PAGE. (D) His_6-Dac was expressed in BL21 cells, as described in Materials and Methods, and purified from 2 mg of lysate over ampicillin Sepharose. The fusion protein was released by four 10-min incubations in ice-cold release buffer. The purified fusion protein was once more captured on fresh ampicillin Sepharose and released in the cold. Aliquots of the lysate (X), the first ampicillin supernatant (S1), the released protein (R1), the second ampicillin supernatant (S2), and the second release (R2) were analyzed by SDS–PAGE (6–20%).

accounting for more than 10% of the soluble protein in the extract (Fig. 5D). In this example, we released the protein on ice. To examine whether PBP5 is functionally damaged during release, we brought the His-Dac to ambient temperature and incubated once more with a fresh batch of ampicillin Sepharose. As shown in Fig. 5D, we captured more than 90% again on ampicillin Sepharose and recovered most of it. This implies that the procedure does not modify the catalytic serine of PBP5 and that this enzyme is indeed a slow β-lactamase, as suggested by others [20,21].

Direct comparison with GST-tag in HEK293 cells

To compare the Dac-tag with the GST-tag, we created mammalian expression vectors (Fig. 6A and B) for either GST- or Dac-tagged SPAK, a protein kinase with important roles in the kidney [22]. We transiently transfected HEK293 cells with the same amount of expression plasmid (5 μg/10-cm dish) and incubated the cells for 2 days, after which we prepared extracts. We immunoblotted the extracts using an antibody to SPAK. SPAK expression levels were similar in both cases, but Dac-SPAK was expressed at slightly higher levels (Fig. 6D). Using the same amount of the appropriate Sepharose, we purified 14 μg of GST-SPAK and 28 μg of Dac-SPAK from two dishes of the transfected cells. We separated 3 μg of the protein, and both the yield and purity of Dac-SPAK exceeded that of GST-SPAK (as shown in Fig. 6C), demonstrating that the Dac-tag is superior to the GST-tag when used in mammalian cells. Interestingly, and in contrast to GST-SPAK, Dac-SPAK bound some β-tubulin, which is not one of the previously seen contaminants, suggesting that tubulin may be a specific SPAK binding partner. In indirect comparisons of GST-cullin Rbx and Dac-cullin Rbx complexes, the Dac-tag outcompeted the GST-tag by a factor 100

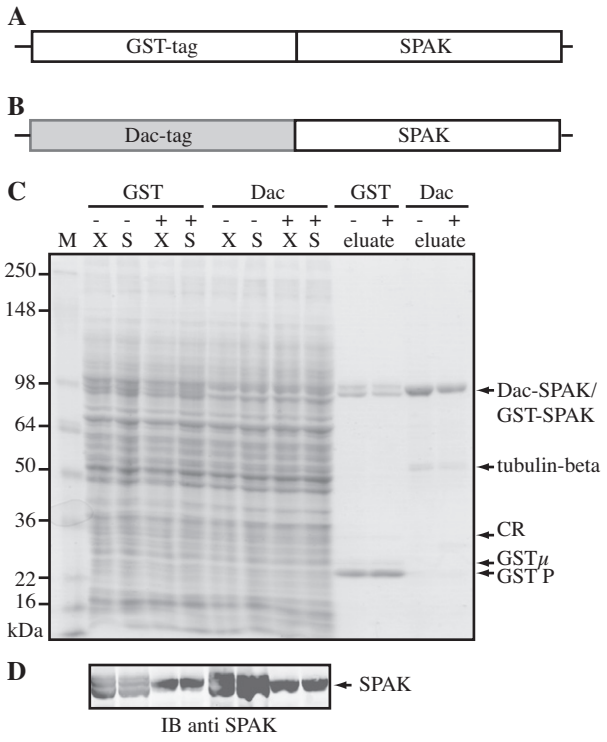

Fig.6. Comparison of Dac-tag with GST-tag in HEK293 cells. (A and B) Mammalian expression vectors were created by cloning GST-SPAK (A) or Dac-SPAK (B) into pcDNA3.1. (C) HEK293 cells were transiently transfected with these vectors. After 2 days, some of the cells were challenged with hypotonic medium (+) to activate SPAK or were left untreated (–). Protein extracts were prepared, and the fusion proteins were purified over either GSH Sepharose or ampicillin Sepharose as appropriate. The Sepharoses were washed with the same wash buffer. GST-SPAK was released by incubation with 10 mM GSH, whereas Dac-SPAK was released by incubating the Sepharose at 4 °C as described above. (D) Here, 10 μg of the lysate from panel C was immunoblotted with a polyclonal SPAK antibody and visualized by a fluorescently labeled anti-sheep antibody using a LICOR infrared scanner.

in yield, probably because the Dac-tag is monomeric and does not cause the formation of large aggregates (not shown).

In summary, although the Dac-tag with a molecular mass of 28.5 kDa is still a relatively large protein, and much of the manipulation is done at ambient temperature, the system may provide a very useful tag for protein production due to its high selectively and the possibility of ligand-free recovery. We find it to be particularly useful for protein expression in insect Sf21 cells and when we purify protein complexes. To remove the tag from the target protein, we clone a TEV site between the tag and the target protein. TEV is very efficient and selective. The contaminants CHC and PR bind at very low levels to ampicillin Sepharose. They may account for 1% of all protein when the target protein expression is very low. We have never detected any contaminants with Sf21 expression. It is not trivial to identify a protein and a ligand that do not bind to undesired proteins, but this system comes very close to that aim. Sometimes, a 28.5-kDa band can be seen in the initial pull-down, representing the Dac-tag without the fusion. This must be due to premature termination of translation. When we are unhappy with the purity of the target protein, we employ a Superdex 75 polishing step, which then always yields extremely pure protein as long as the target protein is not too similar in molecular weight (e.g., 20–45 kDa). Finally, when we try to purify proteins that cannot be purified with other tags because they are unstable or insoluble, we do not, of course, get any improvement with the Dac-tag. Rather, we obtain very little if any protein, in contrast to Ni^{2+} purifications, which always give some contaminants, or GST purifications, which yield cellular GST from eukaryotic cells.

Declaration of competing financial interests

Axel Knebel is the principal owner and managing director of Kinasource, the legal proprietor of the method. Kinasource has applied for worldwide patent protection. The company has invested, and continues to invest, in the method.

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